REMARKS

Claims 1-4, 12, 13, 21-33, 35, 36, 38, and 75-77 were pending when the Office Action of March 21, 2008, was issued. Claims 3, 4, and 21-23 have been cancelled by way of this amendment, and claims 78-85 have been added. The Office rejected claims 1-4, 12, 13, 21-33, 35, 36, 38, and 75-77 under 35 U.S.C. § 112, first paragraph. The Office also rejected claims 1 and 4 under 35 U.S.C. § 102(e) for allegedly being anticipated by U.S. Patent Publication No. 20020048763 ("the 763 publication"). Reconsideration of these rejections is hereby requested.

I. Amendments to the Claims

Claim 1 has been amended to recite that the amino acid sequence of the claimed isolated peptide consists of 8-40 amino acids and comprises the amino acid sequence set forth in any one of SEQ ID NOs: 35-66, wherein the peptide binds to human VEGFR-3. Claim 12 has been amended to further characterize the claimed isolated peptide as comprising the amino acid sequence set forth in any one of SEQ ID NOs: 35-43 and 55-66. The amendments to claims 1 and 12 are supported by the specification at, e.g., page 16, line 8, through page 17, line 10; page 34, line 26, through page 35, line 5; and Table 1 on page 27. Claims 3, 4, and 21-23 have been cancelled, and claims 24, 26, 27, 30, 32, 33, 35, 38, and 76 have been amended to adjust the dependencies of those claims. Claims 78-85 are new. Claim 78 is supported by the specification at, e.g., page 16, lines 3-7. Claim 82 is supported by the specification at, e.g., Table 1 on page 27, and page 14, line 14, through page 15, line 12, which notes that the inventive peptide may comprise the amino acid sequence X_1-X_8 (e.g., amino acid residues 2-9 of SEQ ID NOs: 35 and 55-58), *optionally* further comprising cysteine residues flanking X_1 and X_8 and *optionally* comprising additional residues attached to X_1 or X_8 within the terminal cysteines. Claims 79-81 and 83-85 are supported by the specification at, e.g., page 15, lines 10-12; page 16, lines 17-19; page 17, line 28, through page 18, line 6; and page 34, line 26, through page 35, line 5. No new matter has been added by way of these amendments, and all of the pending claims read on the elected species.

II. The Enablement Rejection Under 35 U.S.C. § 112, First Paragraph, Should Be Withdrawn

Claims 1-4, 12, 13, 21-33, 38, and 75-77 remain rejected under 35 U.S.C. §112, first paragraph, as allegedly not being enabled by the description of the specification. The enablement rejection is respectfully traversed for the reasons set forth below.

To satisfy the enablement requirement of Section 112, first paragraph, an application must contain sufficient information regarding the claimed subject matter as to enable one of ordinary skill to make and use the invention without undue experimentation. See M.P.E.P. § 2164. The Office appears to have rejected the claims for three reasons: (1) the specification assertedly does not disclose the amino acid sequence of the entire 8-100 amino acid peptide; (2) the specification assertedly fails to disclose which amino acids of the "core amino acid sequence" may be substituted while retaining VEGFR-3 binding; and (3) there are no *in vivo* examples demonstrating that the claimed peptides are effective in treating cancer. The Office's second concern is moot in view of the claim amendment reciting specific "core amino acid sequences." The Office's first and third concerns do not render the claims non-enabled because one of ordinary skill could make and use the invention without undue experimentation, and because the Office is applying an improper legal standard for assessing the enablement of a composition claim.

The pending claims, as amended, encompass a limited genus of peptides with specified amino acid length (no larger than 40 amino acids), specified structure (comprising the amino acid sequence of SEQ ID NOs: 34-66 or the amino acid sequence spanning residues 2-9 of SEQ ID NOs: 35 and 55-58), that bind to a specific cell receptor (human VEGFR-3). Applicants provide the core amino acid sequence (i.e., the structure) responsible for the biological activity required by the claims. Because the sequence required for binding is specified in the claim, the enablement requirement is satisfied for peptides containing additional amino acids. In this regard, the claimed peptide sequence is of a finite length such that a *limited* number of peptides are available, and all peptide comprise a core amino acid sequence proven to bind VEGFR-3. Making peptides within the length maximum recited in the claims entails only routine and largely automated techniques to add additional residues *of any sequence* to the core structure of SEQ ID NOs: 34-66. The specification fully discloses methods to make the limited genus of claimed peptides via, e.g., peptide synthesis, phage

display, and other methods well-known in the art (see, e.g., pages 35-41.) The level of skill in the art of peptide synthesis and DNA manipulation is high, and those of ordinary skill in the art have the requisite expertise to generate the claimed limited genus of peptides. In addition, the teaching in the application that a small peptide of the invention is still capable of inhibiting ligand binding to VEGFR-3 when the peptide is expressed as a fusion to glutathione transferase (GST) (a large protein of approximately 26 kDa (see, e.g., Smith and Johnson, *Gene*, *67*, 31-40 (1988)) demonstrates that no undue experimentation would be required to make peptides of 8-40 amino acids that contain the core binding sequence and retain binding activity.

In addition, the specification describes methods to determine candidate peptide binding specificities with respect to VEGFR-3 using, e.g., VEGFR-3 binding assays (see specification at, e.g., page 42, line 1, through page 53, line 2). Screening peptides to, e.g., verify that additional sequence outside the core amino acid sequence did not interfere with the binding activity (already conferred by the core amino acid sequence) is performed quickly using routine techniques, such as those provided in the specification. Routine experimentation, of course, is not undue experimentation. See *In re Wands*, 858 F.2d 731 (Fed. Cir. 1988) (making monoclonal antibodies and screening for binding activity was merely routine screening). It is noteworthy that peptide synthesis and screening is much more routine than monoclonal synthesis and screening, and also that the skill in the art advanced tremendously between the filing date of the Wands application (1980) and the priority date of this application (2001).

The Office also asserted that "the intended use of the peptide is to treat cancer in [a] human," and that "[a] pharmaceutical composition in the absence of *in vivo* working example [is] unpredictable." (Office Action dated March 21, 2008, page 12.) The Office further contends that the pending claims are not enabled because the specification allegedly fails to teach "how to effectively treat any disease such as cancer or reach any therapeutic endpoint in humans by administering peptide." *Id.* Insofar as the Office is requiring *in vivo* proof of efficacy against cancer for *product* claims that do not recite treatment of cancer, the Office commits legal error. The pending claims do not recite a method of treating a disease, nor do the claims require that the claimed peptide treat cancer. The claims recite that the

claimed peptide binds VEGFR-3. (As discussed in detail in the application and summarized below, all VEGFR-3 binding peptides have utilities that extend beyond treatment of cancer.) The application demonstrates that the core amino acid sequences of the peptide bind VEGFR-3 (see, e.g., Table 1 on page 27), and provide several assays to confirm the claimed activity at, e.g., page 42, line 1, through page 53, line 2. Because the application teaches that the peptides bind soluble VEGFR-3 (a molecular entity with demonstrated anti-cancer properties itself (see, e.g., Karpanen et al., *Cancer Res.*, *61*, 1786-90 (March 2001), provided herewith), one enabled utility that would be apparent to a person of ordinary skill is utility for affinity purification of VEGFR-3 Fc.

The application also explicitly discloses several other uses for the claimed peptides, one of which is the treatment or diagnosis of VEGFR-3-related disease (see specification at, e.g., page 53, line 4, through page 68, line 22). Dosages and routes of administration, as well as methods for evaluating anti-tumor activity, are provided (see specification at, e.g., page 49, line 12, through page 51, line 14; and page 70, line 5, through page 74, line 26). However, the specification also discloses several other uses for the claimed peptide, including detecting VEGFR-3, imaging, modulating cell proliferation, and screening for neovascularization (see specification at, e.g., page 17, line 27, through page 18, line 21; page 20, line 21, through page 22, line 2; and page 23, line 20, through page 24, line 22). For example, the specification teaches use of the claimed peptide in tissue imaging techniques to identify expression of VEGFR-3 at, e.g., page 68, line 22, through page 70, line 2. Thus, the specification provides sufficient guidance to enable the ordinarily skilled artisan to use the claimed invention.

The application's teaching that glutathione transferase (GST)-peptide fusions inhibit ligand binding to VEGFR-3 in a cell-based assay supports the explicitly recited utilities (see, e.g., the Example at page 74, line 29, through page 78, line 28). A peptide comprising the amino acid sequence of SEQ ID NO: 35 was linked to GST to create a fusion protein as described at page 77, lines 6-19. The GST portion of the fusion peptide was approximately 26 kDa, i.e., much larger than the longest isolated peptides encompassed by the pending claims (see, e.g., Smith and Johnson, *Gene*, *67*, 31-40 (1988), describing the pGEX-2T vector used in the disclosed experiment). The peptide-GST fusion inhibited

binding of VEGF-C to VEGFR-3 expressed on BaF3 cells as measured by the MTT cell death assay (see specification at, e.g., page 32, lines 4-12; page 78, lines 12-18; and Figure 1). The Example further supports the recited uses of the claimed peptides disclosed in the application.

The Office contends that claims 30 and 32 are not enabled because the specification does not teach which therapeutic protein or antibody (or fragment thereof) is attached to the inventive peptide. The invention does not hinge on the particular therapeutic protein or antibody; the practitioner has the requisite knowledge and skill to select an appropriate therapeutic protein or antibody to fuse or attach to the inventive peptide. Methods of generating chimeric (i.e., fusion) proteins and antibody conjugates are well known in the art (see, e.g., Maniatis et al., *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, New York, 1989; and *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988). Likewise, the specification provides sufficient guidance with respect to modifications that increase the circulating *in-vivo* half-life of the peptide in a mammal, as recited in claim 33. The specification at page 19, lines 12-23, teaches "[s]tandard pharmaceutical and formulation chemistry is used to [increase *in vivo* half-life], e.g., through glycosylation, pegylation, introduction of non-hydrolyzable bonds, mixing with pharmaceutically acceptable diluents, adjuvants, or carriers, and the like." Methods of generating non-hydrolyzable peptides and stable peptide analogs are described in the specification at, e.g., page 32, line 19, through page 33, line 31. The teachings in the application that the claimed peptide when fused to GST (a large protein) is still capable of inhibiting ligand binding to VEGFR-3 demonstrates that no undue experimentation would be required to make the claimed peptides of 8-40 amino acids that retain binding activity. The provided teachings are sufficient to allow one of ordinary skill to make and use the subject matter of claims 30, 32, and 33.

Because Applicants have taught a worker of ordinary skill in the art to make and use the claimed peptides with only routine experimentation, the enablement rejection under 35 U.S.C. § 112, first paragraph, should be withdrawn.

III. The Written Description Rejection Under 35 U.S.C. § 112, First Paragraph, Should Be Withdrawn

The Office rejected claims 1-4, 12, 13, 21-33, 35, 36, 38, and 75-77 under Section 112, first paragraph, for alleged lack of written description. The written description requirement is satisfied when a patent specification describes the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that Applicants had possession of the claimed invention. See, e.g., *Moba, B.V. v. Diamond Automation, Inc.*, 325 F.3d 1306, 1319 (Fed. Cir. 2003). Applicants respectfully traverse the rejection because the application describes the instantly claimed subject matter such that one of ordinary skill could recognize that the applicants invented what is claimed.

The Office maintains that the specification fails to adequately describe the genus of peptides encompassed by the claims. Specifically, the Office asserted that the previous claims lacked sufficient written description because (1) the specification allegedly failed to teach which amino acids within the core sequence could be conservatively substituted while maintaining function; and (2) the previous claims encompassed peptides wherein a large percentage of the amino acids allegedly are unknown. Applicants respectfully disagree with the Office's analysis. The Office continues to fail to appreciate that the core sequence variation is restricted to *conservative* mutations, which are not expected to adversely affect function. In addition, the claimed peptides defined by $X_1X_2X_3X_4X_5X_6X_7X_8$ are quite small in relation to many chemical and biomolecule claims routinely allowed by the Patent Office in accordance with the Office's Written Description Training Manuals (Revision 1; March 25, 2008).

However, solely in an effort to advance prosecution of the application, the claims have been amended to define the core amino acid sequence that confers VEGFR-3 binding function as the amino acid sequence set forth in one of SEQ ID NOs: 35-66, the amino acid sequence set forth in SEQ ID NO: 34, or the amino acid sequence spanning residues 2-9 of SEQ ID NOs: 35 and 55-58. Applicants have provided the partial structure of the claimed isolated peptide, and correlated the disclosed structure with the functional characteristic recited in the claim. The specification describes the claimed invention in sufficient detail to allow one skilled in the art to recognize that the applicants invented what is claimed. Indeed, according to the Office's Guidelines for Examination of Patent

Applications Under the 35 U.S.C. 112, ¶ 1 "Written Description" Requirement, 66 Fed. Reg. 1099 (Jan. 5, 2001), such detailed, relevant identifying characteristics are sufficient to convey that Applicants possessed the currently claimed invention in accordance with Section 112, first paragraph. *Id.* at 1104, 1106; see also *Enzo Biochem, Inc., v. Gen-Probe Inc.*, 323 F.3d 956, 964 (Fed. Cir. 2002).

With regard to the Office's concern that the portion of the peptide outside the core sequence is not adequately described, Applicants have *described the structure associated with function* of the claimed peptides, and the application demonstrates that short peptides, such as the peptide CGYWLTIWGC, can bind VEGFR-3. The claims also have been amended to recite that the isolated peptide consists of 8-40 amino acids. By teaching short peptide sequences and describing a genus of such sequences, Applicants have fulfilled the written description requirement of providing structure generally, and structure associated with function specifically. The application also describes how to make longer peptides that contain the core structure (the "necessary common attributes"), and assay them to confirm that they retain the binding function recited in the claims. The application contemplates that the additional amino acids are optional, and can be any amino acids. Because the written description requirement focuses on the "necessary common attributes" of what is claimed, there is no statutory requirement for describing or defining every additional element or variation that a person of ordinary skill could add to an invention that is described in the application. See M.P.E.P § 2163 ("If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate description requirement is met."). The application also demonstrates that a short peptide core sequence recited in the claims can retain VEGFR-3 binding activity when attached to a significantly longer peptide sequence (GST) in the Example, which is summarized above.

The Office rejected claim 32 because there is allegedly insufficient disclosure about the binding specificity of the antibody and content of the antibody fragment. The invention is not dependent on the particular antibody (or fragment thereof) attached to the peptide, and the specification need only provide sufficient written description to allow one of ordinary skill to recognize that the invention includes attaching the claimed peptide to an

antibody or fragment thereof. See, for example, *In re Herschler*, wherein the C.C.P.A. found that a specification's disclosure of corticosteriod in DMSO was sufficient to support claims drawn to a method of using a mixture of a "physiologically active steroid" and DMSO. 591 F.2d 693, 697 (C.C.P.A. 1979). The court noted that "use of known chemical compounds in a manner auxiliary to the invention must have a corresponding written description only so specific as to lead one having ordinary skill in the art to that class of compounds." *Id.* The general description of peptide conjugates comprising antibodies and fragments thereof at, e.g., page 19, lines 8-11; and page 60, lines 8-30, is sufficient to satisfy the requirements of Section 112, first paragraph.

With respect to claim 33, the Office contends that the application only describes glycosylation and PEGylation and, therefore, the claims must be limited to those modifications. Applicants again point the Office's attention to the specification at page 19, lines 12-23, which teaches "[s]tandard pharmaceutical and formulation chemistry is used to [increase *in vivo* half-life], e.g., through glycosylation, pegylation, introduction of non-hydrolyzable bonds, mixing with pharmaceutically acceptable diluents, adjuvants, or carriers, and the like." Additional description of half-life increasing modifications is found in the specification at, e.g., pages 32-34. The Office has not specifically addressed the teachings of the specification in this regard, and provided no reasoning as to why the disclosure is allegedly inadequate.

For the foregoing reasons, one of ordinary skill in the art would have understood Applicants to be in possession of the invention as presently claimed, so the rejection under 35 U.S.C. §112, first paragraph, for lack of written description should be withdrawn.

IV. The Rejection Under Section 102(e) Should be Withdrawn.

The Office rejected claims 1 and 4 under Section 102(e) for allegedly being unpatentable in view of the '763 publication, which allegedly discloses a peptide comprising a subsequence (GYWITVFG) that falls within the scope of the core amino acid sequence previously recited in claims 1 and 4. The rejection is moot in view of the amendments to the claims. In particular, claim 1 has been amended to define the inventive isolated peptide as comprising the amino acid sequence set forth in any one of SEQ ID NOs: 35-66. SEQ ID

NOs: 35-66 do not comprise the amino acid sequence GYWITVFG. (SEQ ID NO: 34, recited in new claim 78, also does not comprise the amino acid sequence GYWITVFG.) The reference does not teach an isolated peptide comprising an amino acid sequence set forth in SEQ ID NOs: 35-66, and the rejection under Section 102(e) should be withdrawn.

V. Conclusion

In view of the above amendments and remarks, Applicants believe the pending application is in condition for allowance. The Examiner is invited to contact the undersigned attorney by telephone if there are issues or questions that might be efficiently resolved in that manner.

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Respectfully submitted,

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Advances in Brief

Vascular Endothelial Growth Factor C Promotes Tumor Lymphangiogenesis and Intralymphatic Tumor Growth[1]

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Abstract

Many solid tumors produce vascular endothelial growth factor C (VEGF-C), and its receptor, VEGFR-3, is expressed in tumor blood vessels. To study the role of VEGF-C in tumorigenesis, we implanted MCF-7 human breast carcinoma cells overexpressing recombinant VEGF-C orthotopically into severe combined immunodeficient mice. VEGF-C increased tumor growth, but unlike VEGF, it had little effect on tumor angiogenesis. Instead, VEGF-C strongly promoted the growth of tumor-associated lymphatic vessels, which in the tumor periphery were commonly infiltrated with the tumor cells. These effects of VEGF-C were inhibited by a soluble VEGFR-3 fusion protein. Our data suggest that VEGF-C facilitates tumor metastasis via the lymphatic vessels and that tumor spread can be inhibited by blocking the interaction between VEGF-C and its receptor.

Introduction

VEGF-C[4] is a ligand for the lymphatic endothelial receptor VEGFR-3, but it binds also to VEGFR-2, which is the major mitogenic signal transducer for VEGF in blood vascular endothelial cells (1–3). VEGF-C stimulates almost exclusively lymphangiogenesis when applied to differentiated chick chorioallantoic membrane (4) or when overexpressed in the skin of transgenic mice (5). However, more recent studies report that VEGF-C also stimulates angiogenesis in mouse cornea (6), in developing chorioallantoic membrane of chick embryos (6), and in ischemic hind limbs of rabbits (7). Many tumors express VEGF-C, and the expression level has been suggested to correlate with tumor angiogenesis and metastasis via the lymphatic system (8–10). VEGFR-3 is normally expressed predominantly in the lymphatic vessels in adults (11–13), but this receptor is also induced in the angiogenic blood vascular endothelium of many tumors (9, 14, 15). To study the possible effects of VEGF-C on tumor growth, angiogenesis, and lymphangiogenesis, we overexpressed VEGF-C in human MCF-7 breast carcinoma cells, which otherwise produce minimal levels of this growth factor (16). The VEGF-C-overexpressing or vector-transfected cells were then implanted orthotopically and grown as tumors in the mammary fad pads of SCID mice.

Materials and Methods

Plasmid Expression Vectors. The cDNAs coding for the human VEGF-C or $VEGF_{165}$ were introduced into the pEBS7 plasmid (17). The same vector was used for the expression of the soluble receptor chimeras VEGFR-3-Ig, containing the first three immunoglobulin homology domains of VEGFR-3 fused to the Fc-domain of human immunoglobulin γ chain and VEGFR-1-Ig, containing the first five immunoglobulin homology domains of VEGFR-1 in a similar construct (18).

Production and Analysis of Transfected Cells. The MCF-7S1 subclone of the human MCF-7 breast carcinoma cell line was transfected with plasmid DNA by electroporation, and stable cell pools were selected and cultured as described previously (19). The cells were metabolically labeled in methionine and cysteine free MEM (Life Technologies, Inc.) supplemented with 100 μCi/ml [^{35}S]methionine and [^{35}S]cysteine (Redivue Pro-Mix; Amersham Pharmacia Biotech). The labeled growth factors were immunoprecipitated from the conditioned medium using antibodies against VEGF-C (1) or VEGF (MAB293; R & D Systems). The immunocomplexes and the VEGFR-Ig fusion proteins were precipitated using protein A-Sepharose (Amersham Pharmacia Biotech), washed twice in 0.5% BSA, 0.02% Tween 20 in PBS, and once in PBS and analyzed in SDS-PAGE under reducing conditions.

Cell Proliferation and Tumorigenesis Assays. Cells (20,000/well) were plated in quadruplicate in 24-well plates, trypsinized on replicate plates after 1, 4, 6, or 8 days, and counted using a hemocytometer. Fresh medium was provided after 4 and 6 days. For the tumorigenesis assay, subconfluent cultures were harvested by trypsinization and washed twice, and 10^7 cells in PBS were inoculated into the fat pads of the second (axillar) mammary gland of ovarectomized SCID mice, carrying s.c. 60-day slow-release pellets containing 0.72 mg of 17β-estradiol (Innovative Research of America). The ovarectomy and implantation of the pellets were done 4–8 days before tumor cell inoculation. Tumor length and width were measured twice weekly in a blinded manner, and the tumor volume was calculated as the length $\times$ width $\times$ depth $\times$ 0.5, assuming that the tumor is a hemi-ellipsoid and the depth is the same as the width (20).

Histology and Quantitation of the Blood Vessels. The tumors were excised, fixed in 4% paraformaldehyde (pH 7.0) for 24 h, and embedded in paraffin. Sections (7 μm) were immunostained with monoclonal antibodies against PECAM-1 (PharMingen), VEGFR-3 (21), PCNA (Zymed Laboratories), or polyclonal antibodies against LYVE-1 (a kind gift from Dr. David G. Jackson, University of Oxford, Oxford, United Kingdom; Ref. 22), VEGF-C (1) or laminin (a kind gift from Dr. Karl Tryggvason, Karolinska Institute, Stockholm, Sweden) according to published protocols (14). The average of the number of the PECAM-1-positive vessels was determined from three areas ($\times$60) of the highest vascular density (vascular hot spots) in a section. All histological analysis was done using blinded tumor samples.

Adenoviral Expression of Soluble VEGFR-3 and Evan's Blue Draining Assay. The cDNA coding for the VEGFR-3-Ig fusion protein was subcloned into the pAdCMV plasmid, constructed by subcloning the human cytomegalovirus immediate-early promoter, the multiple cloning site, and the bovine growth hormone gene polyadenylation signal from the pcDNA3 (Invitrogen) into the pAdBglII vector, and the adenoviruses were produced as described previously (23). The VEGFR-3-Ig or LacZ control (23) adenoviruses, 10^9

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[4] The abbreviations used are: VEGF-C, vascular endothelial growth factor C; VEGFR, VEGF receptor; SCID, severe combined immunodeficient; PCNA, proliferating cell nuclear antigen; Ig, immunoglobulin.

pfu/mouse, were injected i.v. into the SCID mice 3 h before the tumor cell inoculation. After 3 weeks, four mice from each group were narcotized, the ventral skin was opened, and 5–10 μl of 3% Evan's blue dye (Sigma) in PBS were injected into the tumor. The drainage of the dye from the tumor was followed macroscopically.

Results

Expression of VEGF-C or VEGFR-3-Ig Does Not Affect MCF-7 Cell Growth *in Vitro*. The MCF-7 human breast carcinoma cells were transfected with expression plasmids coding for full-length human VEGF-C or a soluble VEGFR-3 fusion protein (VEGFR-3-Ig), and stable cell pools were selected. For comparison, human $VEGF_{165}$ or VEGFR-1-Ig was expressed in the same cells. The efficient production and secretion of the proteins was verified by immunoprecipitation from the conditioned medium (Fig. 1*A*). However, the growth rate of the transfected cells was not affected (Fig. 1*B*).

VEGF-C Increases Tumor Growth without Affecting Tumor Angiogenesis. The MCF-7 cell pools were implanted into the mammary fad pads of ovariectomized SCID mice carrying slow-release estrogen pellets to provide a constant level of the hormone needed to support the growth of the MCF-7 tumors. Overexpression of VEGF-C increased tumor growth significantly (VEGF-C: 545 mm^3 ± 110 mm^3, control: 268 mm^3 ± 69 mm^3, at 13 days, $n = 8$, $P < 0.0001$, Student's t test; Fig. 2*A*). However, the effect of VEGF-C overexpression on tumor growth was much less dramatic than that of VEGF (VEGF: 1136 mm^3 ± 339 mm^3, control: 189 mm^3 ± 57 mm^3, at 15 days, $n = 6$, $P < 0.0001$, Student's t test; Fig. 2*C*). The increased tumor growth was neutralized by mixing the VEGF-C- or VEGF-overexpressing MCF-7 cells with cells expressing the soluble VEGFR-3 or VEGFR-1 fusion proteins, respectively (Fig. 2, *B* and *D*). The increased growth of the VEGF-C-overexpressing tumors was inhibited also by a circulating soluble VEGFR-3-Ig expressed in the liver by an i.v.-injected recombinant adenovirus (data not shown).

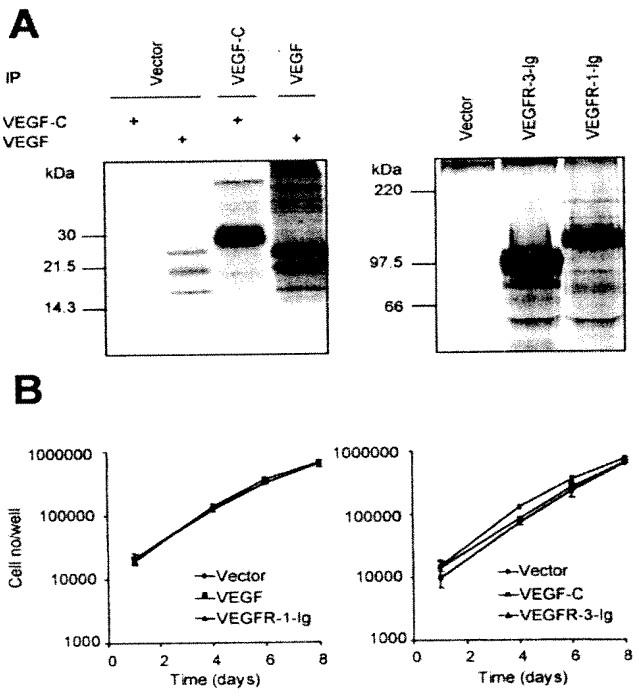

Fig. 1. Overexpression of VEGF-C, VEGF, or the soluble VEGFR-3 or VEGFR-1 fusion proteins does not affect the proliferation of the MCF-7 breast carcinoma cells *in vitro*. *A*, immunoprecipitates of VEGF-C, VEGF, or the soluble receptor proteins from metabolically labeled MCF-7 cells were analyzed in PAGE under reducing conditions. The three polypeptide bands in *Lane 2* represent endogenous VEGF. *B*, cells were seeded in 24-well plates, and after the indicated growth periods, the cells were counted in a hemacytometer. *Bars*, ±SD.

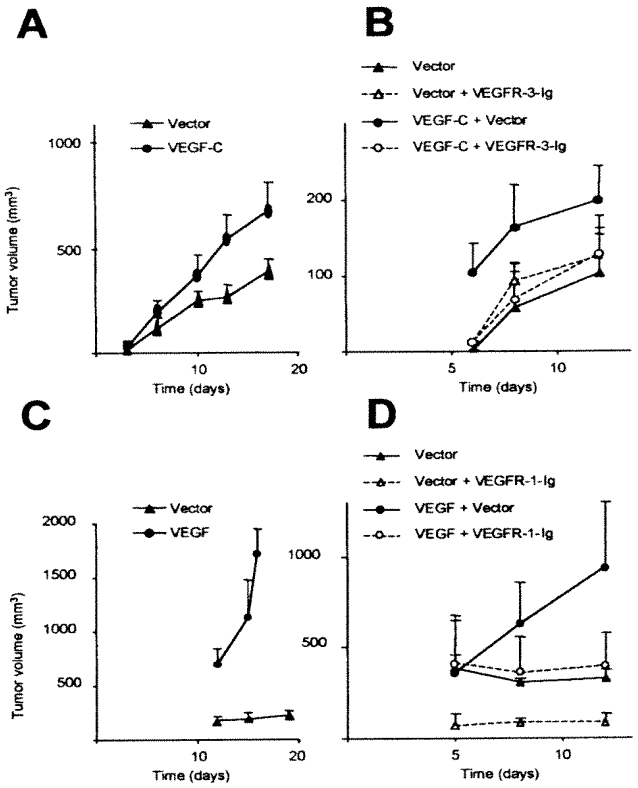

Fig. 2. Effect of VEGF-C, VEGF, and their soluble receptors on tumor growth. *A* and *C*, 10^7 MCF-7 cells stably transfected with VEGF-C (*A*), VEGF (*C*), or empty vector were injected into the mammary fat pads of ovarectomized SCID mice carrying slow-release estrogen pellets. *B*, equal numbers of MCF-7 cells stably transfected with VEGF-C, VEGFR-3-Ig, or empty vector were mixed and injected into the mammary fat pads. *D*, equal numbers of MCF-7 cells stably transfected with VEGF, VEGFR-1-Ig, or empty vector were mixed and injected into the mammary fat pads. The diameters of the formed tumors were measured at the indicated times, and the tumor volumes were calculated. *Bars*, ±SD.

To study the effect of VEGF-C on tumor angiogenesis, sections of the tumors were stained for PECAM-1, an endothelial antigen expressed primarily in blood vessels and only weakly in lymphatic vessels. Quantitation of the PECAM-1-positive vessels in the tumors revealed that overexpression of VEGF-C had very little effect on the density of the tumor blood vessels (40.2 ± 12.2 vessels/microscopic field for VEGF-C tumors, $n = 18$, and 36.6 ± 11.6 for control tumors, $n = 23$; average of three different experiments; Fig. 3). In contrast, overexpression of VEGF increased the vascular density ~2-fold (Fig. 3).

VEGF-C Overexpression Is Associated with Lymphangiogenesis and Intralymphatic Growth of Tumor Cells. The effect of VEGF-C on tumor-associated lymphatic vessels was analyzed by immunostaining for the lymphatic specific marker LYVE-1 (22). This marker revealed highly hyperplastic lymphatic vessels in the periphery of the VEGF-C-overexpressing tumors (Fig. 4*A*). The proliferating cell nuclear antigen was detected in many of the LYVE-1-positive endothelial cells (Fig. 4*A*, *inset*), showing that these lymphatic vessels were actively proliferating. Confirmation of the lymphatic identity of the vessels was obtained by staining for VEGFR-3 (Fig. 4*C*) and by the lack of staining for the basal lamina component laminin (data not shown). Thin lymphatic vessels were also present inside some of the VEGF-C-overexpressing tumors (Fig. 4*B*).

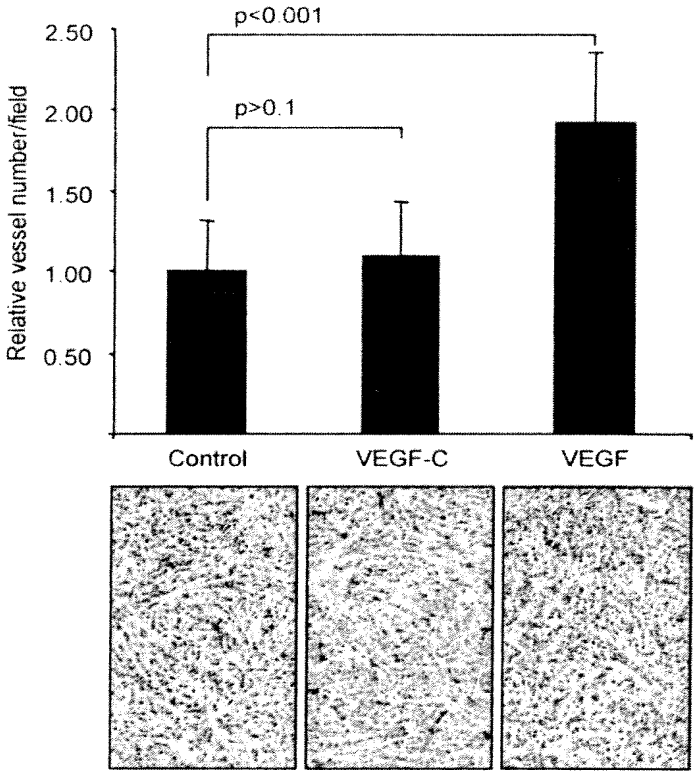

Fig. 3. Tumor angiogenesis is not affected by overexpression of VEGF-C. Sections of VEGF-C- or VEGF-overexpressing tumors or of control tumors were stained for the endothelial cell marker PECAM-1 (*bottom panel*). The positive vessels in three microscopic fields of the highest vascular density were counted and normalized to the number of vessels in the control tumors (*top panel*). Note that whereas VEGF induced an ~2-fold increase in vascular density, VEGF-C had no significant effect on tumor blood vessels. The relative vessel numbers were compared by the Student's *t* test with two-tailed distribution and two-sample equal variance. *Bars*, ±SD.

The lymphatic vessels in the tumor periphery were commonly infiltrated by the VEGF-C-positive tumor cells (Fig. 4, *A*, *C*, and *D*). In a striking contrast, the VEGF-overexpressing and control tumors contained no or only few lymphatic vessels (Fig. 4, *E* and *F*).

VEGF-C-induced Lymphangiogenesis Is Inhibited by a Circulating Soluble VEGFR-3 Fusion Protein. In human breast cancer, the sentinel node method is used to trace lymphatic drainage and metastatic spread (reviewed in Ref. 24). To trace lymphatic drainage of the MCF-7 tumors, Evan's blue dye was injected into VEGF-C-overexpressing or control tumors in mice infected with VEGFR-3-Ig or control adenovirus. Control experiments indicated that infection of cultured human embryonic kidney cells with the VEGFR-3-Ig adenovirus resulted in the secretion of high amounts of the soluble VEGFR-3-Ig fusion protein, and i.v. infection of mice led to high systemic levels of the VEGFR-3-Ig fusion protein in the serum.[5] Injection of Evan's blue dye into the tumors resulted in the staining of lymphatic but not blood vessels and revealed an increased number of enlarged lymphatic vessels surrounding the VEGF-C-overexpressing tumors (Fig. 4*G*) when compared with control tumors (Fig. 4*H*). Most of the enlarged lymphatic vessels were absent from VEGF-C-overexpressing tumors in mice treated with the VEGFR-3-Ig adenovirus (Fig. 4*I*). These results were confirmed by immunohistochemical analysis of the tumor samples (data not shown).

Discussion

This study shows that VEGF-C overexpression in MCF-7 mammary tumors strongly and specifically induces the growth of tumor-associated lymphatic vessels but does not have major effects on tumor angiogenesis. However, tumor growth was significantly increased by VEGF-C overexpression. Furthermore, increased tumor growth and tumor-associated lymphangiogenesis were inhibited by a soluble VEGFR-3 fusion protein. On the other hand, VEGF-overexpressing and control tumors almost completely lacked lymphatic vessels.

Because of the lack of specific markers, it has been questioned whether tumors can actively induce lymphangiogenesis or if solid tumors just encompass by overgrowth the already existing lymphatic vessels and compress them because of the high interstitial fluid pressure inside the tumor. In various experimental models, the latter seems to be the case (25, 26). Here, for the first time, we show that overexpression of VEGF-C can induce the growth of lymphatic vessels in association with experimental tumors. The VEGF-C-induced lymphatic vessels in the tumor periphery were highly hyperplastic and mostly filled with tumor cells, whereas the lymphatic vessels inside the tumor were flattened and in general without a lumen. These intratumoral lymphatic vessels may be trapped by multiple expanding tumor cell islets in tumor xenografts, but they may be rare in naturally occurring tumors. Unlike lymphatic endothelial cells in normal adult tissues, the lymphatic endothelial cells associated with the MCF-7 tumors were actively proliferating. On the basis of this information, we speculated that most of the peri- and intratumoral lymphatic vessels were generated by proliferation of the endothelial cells of preexisting lymphatic vessels.

Although the spread of cancer through the lymphatics into the regional lymph nodes has long been an important prognostic indicator in clinical use, tumor metastasis at the mechanistic level is still poorly understood. The growth of tumor cells inside the enlarged lymphatic vessels associated with the VEGF-C-overexpressing tumors in this study resembles the peritumoral lymphatic invasion, which correlates with metastatic spread to the lymph nodes and poor survival in human breast cancer (27). This suggests that expression of VEGF-C can promote tumor metastasis via the lymphatic system. Despite this, we did not detect macroscopic metastases in the lymph nodes of mice bearing the VEGF-C-overexpressing tumors (data not shown). This may be attributable to the facts that MCF-7 tumors rarely form macrometastasis (28) and that the duration of our experiments was relatively short. However, lymph node micrometastases were promoted by VEGF-C overexpression in the MCF-7 tumors.[6]

VEGF and its receptor VEGFR-2 are considered to be the main regulators of tumor angiogenesis (2, 3). Also VEGFR-3, although normally restricted to the lymphatic endothelial cells in adults, is up-regulated in the blood vessels of many kinds of solid tumors (9, 14). A previous report suggested that VEGFR-3 could be involved in the maintenance of the integrity of the endothelial cell lining during tumor angiogenesis (21). Therefore, we speculated that VEGF-C may influence tumor neovascularization. However, in the present tumor model, overexpression of VEGF-C, in comparison with VEGF, did not significantly increase tumor angiogenesis. Instead, its effects were mainly lymphangiogenic.

The increased growth of the primary tumors overexpressing VEGF-C was unexpected, given that VEGF-C had no effect on tumor cell proliferation in cell culture or on tumor angiogenesis. The effect of VEGF-C on tumor growth was not simply attributable to variation between the cell pools, as shown by the ability of the VEGFR-3 fusion protein to inhibit the growth of VEGF-C-overexpressing tumors. By

[5] T. Karpanen and T. Makinen, unpublished data.

[6] M. Mattila, J. Ruohola, and C. P. Härkönen, unpublished data.

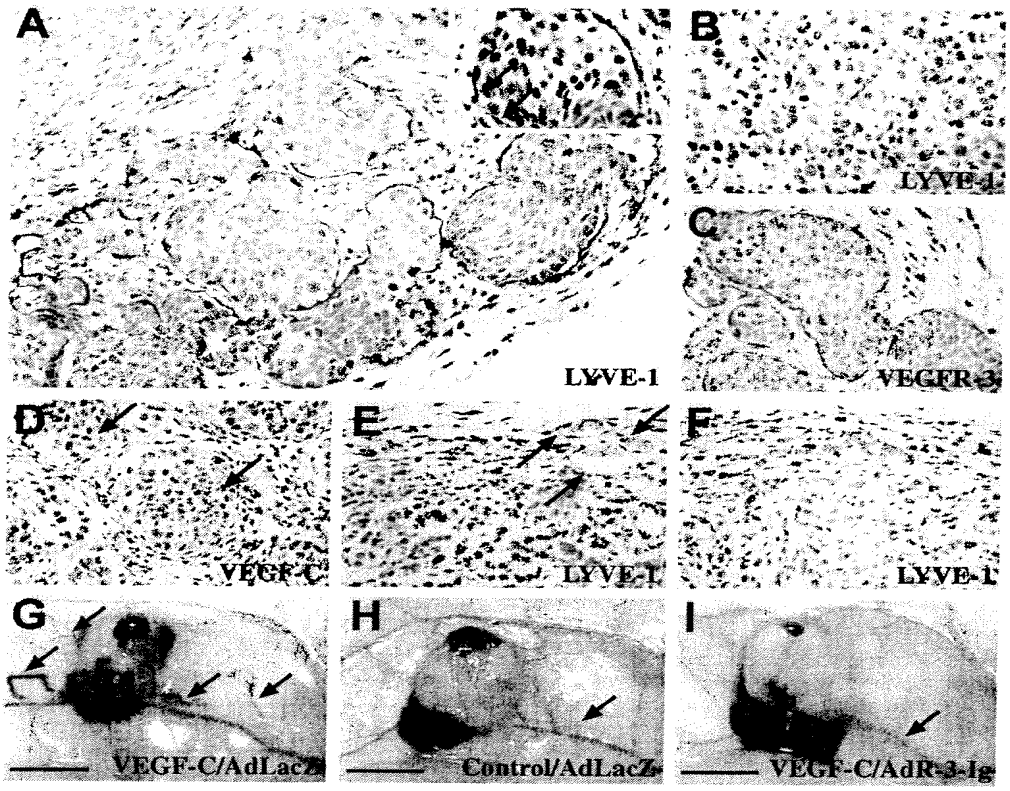

Fig. 4. Overexpression of VEGF-C, but not VEGF, induces tumor-associated lymphangiogenesis. *A*, hyperplastic LYVE-1-positive (*red*) lymphatic vessels in the periphery of VEGF-C-overexpressing tumors are frequently infiltrated by tumor cells. *Inset* in *A*, double staining for LYVE-1 (*red*) and PCNA (*brown*). Note that some LYVE-1-positive cells are also labeled for PCNA (*arrows*). *B*, intratumoral lymphatic vessels appear flattened and without a lumen. *C*, VEGFR-3 (*red*) and *D*, VEGF-C (*red*) staining of sections from VEGF-C-overexpressing tumors. Note that the tumor cells growing inside the lymphatic vessels produce more VEGF-C than the rest of the tumor (*arrows* in *D*). *E* and *F*, LYVE-1 staining of sections from VEGF-overexpressing and control tumors, respectively. *Arrows* in *E*, LYVE-1-negative blood vessels. All sections were counterstained with hematoxylin (*blue*). *G* and *H*, drainage of Evan's blue dye from a VEGF-C-overexpressing tumor and from a control tumor in mice treated with LacZ adenovirus, respectively. *I*, drainage of Evan's blue dye from a VEGF-C-overexpressing tumor in a mouse treated with VEGFR-3-Ig adenovirus. The tumors shown in *G–I* were selected to be of similar size and were photographed 6 min after injection of Evan's blue. *A* and *B*, ×200; *C–F*, ×120. *Bars* in *G–I*, 0.5 cm.

injecting Evan's blue dye into the tumors, we observed that an increased number of large draining lymphatic vessels were associated with the VEGF-C-overexpressing tumors. One could speculate that the higher number of functional lymphatic vessels may result in a better lymphatic drainage and thus a lower interstitial pressure and enhanced blood perfusion of the VEGF-C-overexpressing tumors.

In conclusion, our results show that VEGF-C produced by tumor cells can induce the growth of lymphatic vessels around tumors and thus facilitate the intralymphatic spread of cancer. Because of the specific lymphangiogenic response and the lack of significant effects on tumor angiogenesis, the VEGF-C-overexpressing MCF-7 breast carcinoma represents a useful model to study the development of tumor-associated lymphatic vessels. Furthermore, the data suggest that inhibition of tumor-associated lymphangiogenesis, for example by gene therapy using soluble VEGFR-3 proteins, could be a valuable way of inhibiting tumor metastasis.

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Note Added in Proof

While this paper was being processed, three other papers have also reported increased tumor lymphangiogenesis in different VEGF-C or VEGF-D overexpressing tumor models (Stacker *et al.* Nature Medicine, *7:* 186–191, Skobe *et al.* Nature Med., *7:* 192–198, Mandriotta *et al.* EMBO J, in press). In these models, also lymphatic metastasis was enhanced. Furthermore, Kadambi *et al.* (Cancer Res., in press, 2001) report that in the early stages of tumorigenesis, VEGF-C can increase tumor angiogenesis as well.

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Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase

(Affinity chromatography; expression vector; plasmid; *Plasmodium falciparum*; protease; recombinant DNA; *Schistosoma japonicum*)

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SUMMARY

Plasmid expression vectors have been constructed that direct the synthesis of foreign polypeptides in *Escherichia coli* as fusions with the C terminus of Sj26, a 26-kDa glutathione *S*-transferase (GST; EC 2.5.1.18) encoded by the parasitic helminth *Schistosoma japonicum*. In the majority of cases, fusion proteins are soluble in aqueous solutions and can be purified from crude bacterial lysates under non-denaturing conditions by affinity chromatography on immobilised glutathione. Using batch wash procedures several fusion proteins can be purified in parallel in under 2 h with yields of up to 15 μg protein/ml of culture. The vectors have been engineered so that the GST carrier can be cleaved from fusion proteins by digestion with site-specific proteases such as thrombin or blood coagulation factor X_a, following which, the carrier and any uncleaved fusion protein can be removed by absorption on glutathione-agarose. This system has been used successfully for the expression and purification of more than 30 different eukaryotic polypeptides.

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Abbreviations: Ap, ampicillin; bp, base pair(s); factor X, blood coagulation factor which is activated to factor X_a by Russell's viper venom; GMCSF, granulocyte-macrophage colony stimulating factor; GST, glutathione *S*-transferase; IgG, immunoglobulin G; IPTG, isopropyl-β-D-thiogalactopyranoside; kb, 1000 bp; MTPBS, 150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4 (pH 7.3); nt, nucleotide(s); *ori*, origin of DNA replication; pGEX, plasmid expression vector(s); PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; ^{R}, resistance; RESA, ring-infected erythrocyte surface antigen; SDS, sodium dodecyl sulfate; Sj26, M_r 26000 GST of *S. japonicum*; Tc, tetracycline; X_a blood coagulation factor X_a.

INTRODUCTION

Several vectors have been constructed that simplify the purification of foreign polypeptides expressed in *E. coli* (reviewed by Marston, 1986). For example, polypeptides expressed as fusions with *E. coli* β-galactosidase (Gray et al., 1982; Koenen et al., 1982; Rüther and Müller-Hill, 1983) can be purified from crude cell lysates by substrate-affinity or immuno-affinity chromatography (Germino et al., 1983; Ullmann, 1984; Promega Biotec) or if, like *trpE* fusion proteins, they are insoluble (Itakura et al., 1977; Kleid et al., 1981; Young and Davis, 1983; Stanley and Luzio, 1984) they can be purified from the insoluble fraction of lysed bacteria (Marston, 1986). Other vectors direct the synthesis of polypeptides as fusions with staphylococcal protein A that can be purified by affinity chromatography on IgG-Sepharose (Uhlen et al., 1983; Nilsson et al., 1985; Abrahmsen et al., 1986; Lowenadler et al., 1986). A disadvantage of these methods is that the denaturing reagents used during purification can be expected to alter the antigenicity and functional activity of the purified product, while an additional problem with the protein A system is that the binding of fusion proteins to IgG complicates immunological analysis. Alternative purification strategies involve the synthesis of polypeptides containing poly-arginine at their C terminus that can be purified by cation-exchange chromatography (Sassenfeld and Brewer, 1984), or the production of polypeptides that are secreted into the periplasmic space or culture medium (Marston, 1986; Abrahmsen et al., 1986; Lowenadler et al., 1986; Kato et al., 1987). Although these last approaches have been used successfully in some instances, their generality is unclear.

We describe here novel pGEX based on the enzyme glutathione GST (EC 2.5.1.18) that avoid several of the difficulties described above. We have previously identified an M_r 26000 antigen (Sj26) encoded by the parasitic helminth *S. japonicum* as a GST (Smith et al., 1986) and have expressed enzymatically active Sj26 in *E. coli* (Smith et al., 1988). Many mammalian GST isozymes can be purified by affinity chromatography on immobilised glutathione followed by competitive-elution with excess reduced glutathione (Simons and Vander Jagt, 1977; Mannervik, 1985), and this property is shared by both native Sj26 and Sj26 synthesised in *E. coli* (Smith et al., 1986; 1988). This last observation suggested to us that it might be possible to develop an expression vector that would direct the synthesis of foreign polypeptides as fusions with Sj26 that could then be purified under non-denaturing conditions by glutathione-affinity chromatography. We show here that a variety of eukaryotic polypeptides can be expressed in *E. coli* as soluble and stable GST fusion proteins and that these fusion proteins can be readily purified.

MATERIALS AND METHODS

(a) Affinity purification of fusion proteins

Overnight cultures of *E. coli* transformed with parental or recombinant pGEX plasmids were diluted 1:10 in 800 ml of fresh medium and grown for 1 h at 37 °C before adding IPTG to 0.1 mM. After a further 3–7 h of growth, cells were pelleted and resuspended in 1/50–1/100 culture volume of MTPBS. Cells were lysed on ice by mild sonication and after adding Triton X-100 (BDH Chemicals) to 1%, were subjected to centrifugation at 10000 × *g* for 5 min at 4 °C. The supernatant was mixed at room temperature in a 50-ml polypropylene tube on a rotating platform with 1–2 ml 50% glutathione-agarose beads (sulphur linkage, Sigma). After absorption for 2 min, beads were collected by brief centrifugation at 500 × *g* and washed three times with 50 ml MTPBS. Fusion protein was eluted by competition with free glutathione using 2 × 2-min washes with 1 bead volume of 50 mM Tris · HCl (pH 8.0) containing 5 mM reduced glutathione (Sigma) (final pH 7.5, freshly prepared). Beads were preswollen in MTPBS, washed twice in the same buffer and stored in MTPBS at 4 °C as a 50% solution (v/v). Contamination of purified fusion proteins with *E. coli* proteins, is reduced by including Triton X-100 during absorption with glutathione-agarose and also by minimising the period of sonication. The yield of unstable fusion proteins can be increased by delaying the addition of IPTG until less than an hour before cell harvest. Glutathione-agarose beads have a capacity of at least 8 mg of fusion protein/ml of swollen beads, and can either be used several times

for the same fusion protein or else recycled by washing with 3 M NaCl (Simons and Vander Jagt, 1981). Yields of fusion protein were calculated from the absorbance at 280 nm using the relation 1 A_{280} = 0.5 mg/ml derived from protein concentration estimations (Hartree, 1972) of protein purified from cells transformed with pGEX-1 and using bovine serum albumin as a standard.

(b) Screening of transformants

Mass screening of transformants for expression of fusion protein was conveniently carried out on 1.5 ml culture resuspended in 300 μl MTPBS. After sonication and centrifugation, the supernatant was mixed with 50 μl of 50% glutathione-agarose beads, washed with 3 × 1 ml MTPBS and the beads were boiled in 100-μl sample buffer for analysis on a 0.1% SDS–10% polyacrylamide gel (Laemmli and Favre, 1973) followed by staining with 0.05% Coomassie blue.

RESULTS AND DISCUSSION

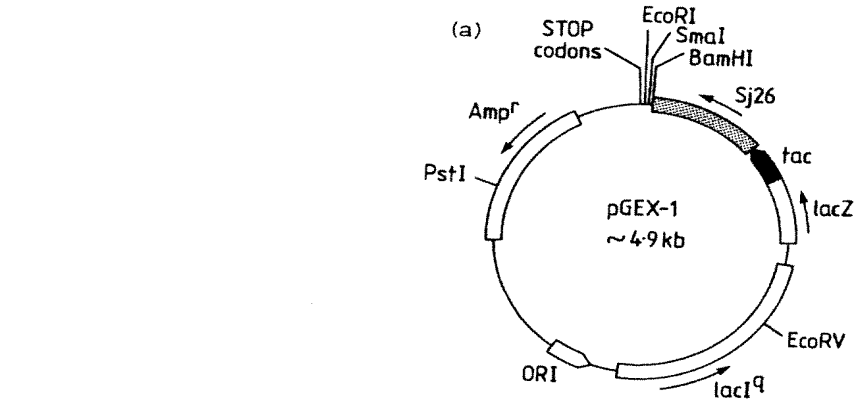
(a) Construction and structure of the pGEX vectors

The plasmid pSj5 directs the synthesis of Sj26 in *E. coli* under the control of the IPTG-inducible *tac* promoter (Smith et al., 1988). Through a series of manipulations pSj5 was modified so that foreign polypeptides could be expressed as fusions with the C terminus of Sj26. The resulting plasmid (pGEX-1) (Fig. 1) contains: (*i*) the *tac* promoter (Amann et al., 1983; De Boer et al., 1983a) followed by the complete coding sequence of Sj26 (Smith et al., 1986; 1987) in which the normal termination codon is replaced by a polylinker containing unique recognition sites for *Bam*HI, *Sma*I and *Eco*RI and followed by TGA translation termination codons in all three reading frames (Fig. 1b); (*ii*) the β-lactamase-coding gene Ap^R; (*iii*) *ori*; and (*iv*) a fragment of the *lac* operon containing the over-expressed *lacI*^{q} allele of the *lac* repressor and part of *lacZ*. Two derivatives of pGEX-1 were constructed (pGEX-2T and pGEX-3X; Fig. 1b) in which the reading frame at the multiple cloning site is shifted by either one or two nt through the introduction of oligodeoxynucleotides encoding the cleavage-recognition sequences of the site-specific proteases thrombin (pGEX-2T) or factor X_a (pGEX-3X).

Induction of the *tac* promoter with IPTG in cells transformed with pGEX-1 results in the synthesis of a 27.5-kDa polypeptide consisting of Sj26 with an additional 10-aa residue at its C terminus (Fig. 2a). Despite its abundance, the 27.5-kDa polypeptide does not form insoluble inclusion bodies and remains in the supernatant of cells lysed by sonication and subjected to centrifugation at 10000 × *g* for 5 min (Fig. 2b). Furthermore, the C-terminal extension to Sj26 does not affect binding to glutathione-agarose and so affinity chromatography of cell extracts results in the efficient purification of the 27.5-kDa molecule with yields of at least 15 μg/ml of culture and in the absence of detectable contamination with *E. coli* proteins (Fig. 2b). Similar properties are observed for the modified Sj26 polypeptides encoded by pGEX-2T and pGEX-3X that both contain an additional 14-aa residue at the C terminus. In the absence of inducer, the plasmid-encoded *lacI*^{q} allele is efficient in repressing transcription from the *tac* promoter (Fig. 2a), even in *E. coli* strains such as C600 or GM48 (Marinus, 1973) that carry a wild-type *lacI* allele (unpublished data).

(b) Expression and purification of *Plasmodium falciparum* antigens

To test the generality of the pGEX vectors as a system for the expression and purification of foreign polypeptides, cDNAs corresponding to several different antigens of *P. falciparum*, the causative agent of falciparum malaria, were inserted into the multiple cloning site of the appropriate pGEX vector. The cDNAs chosen encode portions of two different antigens (Ag63, Ag361) both related to heat-shock protein 70 (Bianco et al., 1986; Peterson et al., 1987), and two antigens containing tandemly repeated peptides (Ag16, *Eco*RI-RESA) (Coppel et al., 1983; Favaloro et al., 1986). In each case synthesis of an abundant GST fusion protein was observed and these fusion proteins could be purified by affinity chromatography of cell extracts on immobilised glutathione with yields of between 1.6 and 5 μg/ml of culture (Fig. 3). Of 21 different *P. falciparum* cDNAs or cDNA fragments that have been expressed in the



(b)

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                Pro  Lys  Ser  Asp  Pro  Arg  Glu  Phe  Ile  Val  Thr  Asp  ***
pGEX-1          CCA  AAA  TCG  GAT  CCC  CGG  GAA  TTC  ATC  GTG  ACT  GAC  TGA  CGA  TCT  G
                                   BamHI     SmaI     EcoRI

                                        Thrombin
                Pro  Lys  Ser  Asp  Leu  Val  Pro  Arg↓ Gly  Ser  Pro  Gly  Ile  His  Arg  Asp  ***
pGEX-2T         CCA  AAA  TCG  GAT  CTG  GTT  CCG  CGT  GGA  TCC  CCG  GGA  ATT  CAT  CGT  GAC  TGA  CTG  ACG  ATC  TG
                                                  BamHI     SmaI     EcoRI

                                   Factor X
                Pro  Lys  Ser  Asp  Leu  Ile  Glu  Gly  Arg↓ Gly  Ile  Pro  Gly  Asn  Ser  Ser  ***
pGEX-3X         CCA  AAA  TCG  GAT  CTG  ATC  GAA  GGT  CGT  GGG  ATC  CCC  GGG  AAT  TCA  TCG  TGA  CTG  ACT  GAC  GAT  CTG
                                             BamHI     SmaI     EcoRI
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Fig. 1. Structure of the pGEX vectors. (**a**) Schematic representation of pGEX-1. The positions of unique *Pst*I and *Eco*RV restriction sites are indicated. (**b**) Nucleotide sequence of pGEX-1, pGEX-2T and pGEX-3X at the C terminus of the Sj26 cDNA. The normal translation termination codon of the Sj26 cDNA began at nt 7 and has been destroyed through the introduction of oligodeoxynucleotides encoding cleavage sites for *Bam*HI, *Sma*I and *Eco*RI (underlined by brackets) and TGA stop codons in all three frames (underlined). The vectors pGEX-2T and pGEX-3X contain additional sequences encoding protease cleavage sites recognised by thrombin and factor X_a, respectively. Details of the construction of these vectors are as follows. Multiple cloning sites were created in the pSj1 Sj26 cDNA (Smith et al., 1986; 1988) through the introduction of a *Bam*HI linker at the unique *Mnl*I cleavage site so that the TAA translation termination codon of Sj26 was replaced with the sequence TCGGATCC. The 5′ terminus of the pSj1 cDNA was also altered through the replacement of the 5′-terminal *Eco*RI-*Sau*96i fragment with oligodeoxynucleotides containing a *Bam*HI cleavage site followed by the sequence CACCATGTCC and then nt 12–38 of the pSj1 cDNA, so producing a *Bam*HI fragment encoding native Sj26 (Smith et al., 1988). This *Bam*HI fragment was inserted into the *Bam*HI site of pIC19H (Marsh et al., 1984) such that the cDNA 3′ terminus was followed by unique *Sma*I, *Eco*RI, *Cla*I and *Eco*RV cleavage sites. A blunt-ended *Bam*HI-*Eco*RV fragment containing the reconstructed Sj26 cDNA was inserted into the *Pvu*II site of p*tac*12ΔEco [p*tac*12 (Amann et al., 1983) modified by filling in the unique *Eco*RI site and religation] in the correct orientation for transcription from the *tac* promoter. This plasmid (pSj10) was further modified through the introduction of an oligodeoxynucleotide (5′–TGACTGACTGA–3′) encoding stop codons in all three frames into the blunt-ended *Cla*I site at the cDNA 3′ terminus, while the *Bam*HI cleavage site at the cDNA 5′ terminus was deleted by filling in using PolIk. Cells transformed with this plasmid (pSj10ΔBam7Stop7) and induced with IPTG synthesised a 27.5-kDa polypeptide but at less than 20% of the level in cells transformed with pSj5, a plasmid derived from p*tac*11 encoding native Sj26 (Smith et al., 1988). This difference in expression may be due to the increased G + C content and length of the region between the *tac* ribosome-binding site and the ATG translation initiation codon in pSj10ΔBam7Stop7 (Stormo et al., 1982; De Boer et al., 1983b) and so the 3′ terminus of the modified Sj26 cDNA in pSj10ΔBam7Stop7 containing the multiple cloning sites and termination codons was introduced into pSj5 as follows. A *Hin*dIII-*Nde*I fragment of pSj5 containing the gene for Tc^R was replaced with a 1.7-kb *Eco*RI fragment derived from pMC9 (Miller et al., 1984) containing the *lacI*^{q} allele and a portion of *lacZ*. Blunt-end ligation of purified fragments after treatment with PolIk produced a plasmid (p4.5) in which *lacI*^{q}, Ap^R and *tac*-Sj26 are all transcribed in the same direction. The *Eco*RI cleavage site at the 3′ terminus of the Sj26 cDNA in p4.5 was removed by filling in and religation while the *Eco*RI site at the cDNA 5′ terminus was destroyed by mutagenesis as described by Mandecki (1986) using a 30-mer oligodeoxynucleotide to generate the sequence *tac*-GTATTC-Sj26 cDNA. A *Bcl*I fragment of this plasmid containing the 3′ terminus of *lacI*^{q}, the *tac* promoter and the 5′ portion of the Sj26 cDNA was inserted into the unique *Bcl*I site of a plasmid formed by joining a *Bcl*I-*Eco*RI fragment of p4.5 containing Ap^R, *ori* and the 5′ portion of *lacI*^{q}

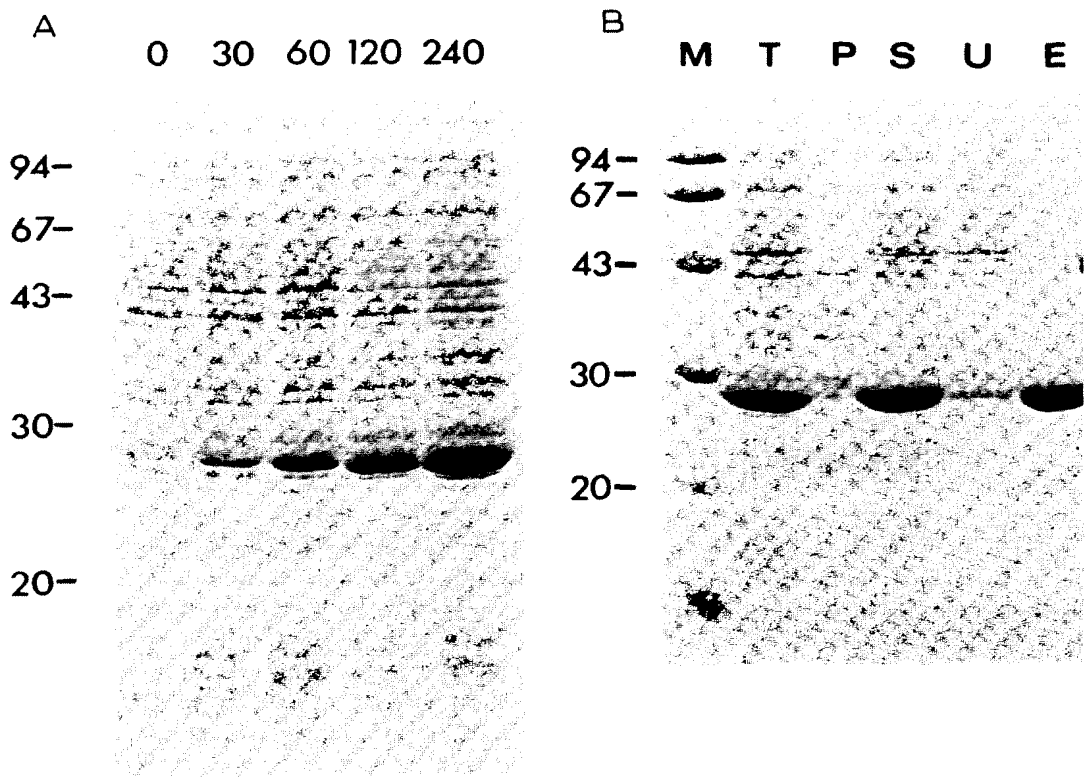

Fig. 2. Expression and purification of GST in cells transformed with pGEX-1. (A) Timecourse of expression after induction. An overnight culture of cells transformed with pGEX-1 was diluted 1:10 in fresh medium, grown for 90 min and IPTG added to 1 mM. Samples were taken at the times indicated and separated by electrophoresis through a 0.1% SDS–13% polyacrylamide gel (Laemmli and Favre, 1973) followed by staining with Coomassie blue. (B) Purification of GST. Cells transformed with pGEX-1 were grown as above except IPTG was added to 0.1 mM and the culture was harvested 3 h after induction. GST was purified as described in MATERIALS AND METHODS, section **a**, and samples taken of whole cells (T), insoluble pellet (P) and supernatant (S) after centrifugation, unbound material (U) after incubation of supernatant with glutathione-agarose beads and purified material (E) eluted from beads. Samples were equivalent to 200 μl of culture and were analysed as described above. The position and sizes (kDa) of molecular weight markers (M) are indicated.

pGEX vectors, 14 have given rise to soluble or partially soluble fusion proteins that could be purified by affinity chromatography on immobilised glutathione (our unpublished data; L. Corcoran, A. Cowman, P. Crewther, C. Langford, V. Marshall, G. Peterson and J. Smythe, personal communications). An even higher success rate (22/26) has been observed for the expression of cDNAs derived from other organisms including those encoding portions of parasite antigens (unpublished data), human auto-antigens (J.

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and a *Bcl*I-*Acc*I fragment of pSj10DBam7Stop7 containing the 3′ terminus of the Sj26 cDNA followed by multiple cloning sites, termination codons and nt 2067–2246 of pBR322. Cleavage with *Bcl*I was on plasmid DNA grown in methylase-deficient GM48 cells (Marinus, 1983) and the *Eco*RI and *Acc*I termini were blunt-ended by treatment with PolIk. A transformant was identified containing a plasmid (pGEX-1) with the structure shown in Fig. 1a. Oligodeoxynucleotides encoding cleavage recognition sites of thrombin or factor X_a were inserted into the *Bam*HI site of pGEX-1 generating plasmids (pGEX-2T and pGEX-3X) in which the unique *Bam*HI site is frame-shifted by one or two nucleotides (Fig. 1b). Nucleotide sequences were confirmed by dideoxynucleotide sequencing of plasmid DNA (Chen and Seeburg, 1985) and, except where indicated, plasmids were transformed into *E. coli* strain JM109 (Yanisch-Perron et al., 1985). Restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs and used according to the manufacturer's instructions. (Amp^r = Ap^R; ORI = *ori*.)

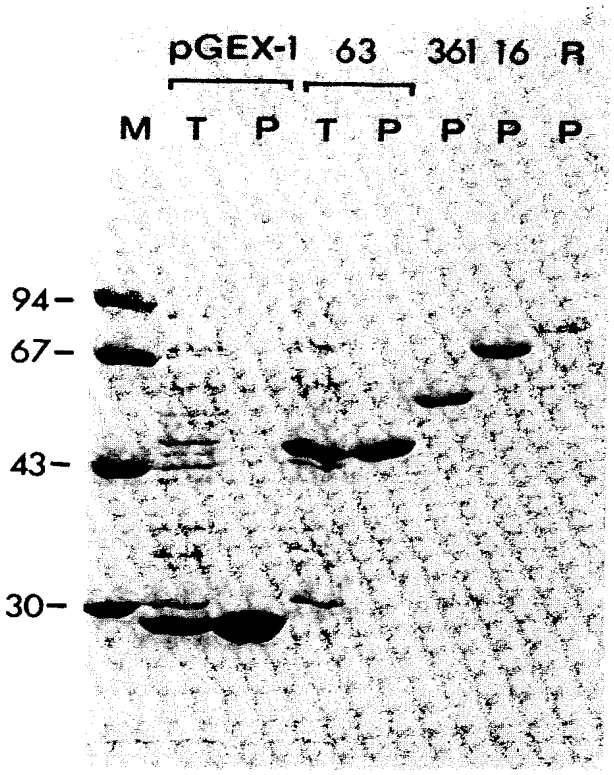

Fig. 3. Purification of *P. falciparum* antigens expressed as GST fusion proteins. Growth of cell cultures and purification of fusion proteins was as described in MATERIALS AND METHODS, section **a**, and samples were analysed by electrophoresis through a 0.1% SDS–10% polyacrylamide gel followed by staining with Coomassie blue. T, total cell extract; P, material purified on glutathione-agarose. Cells were transformed with pGEX-1, or recombinant plasmids consisting of a 555-bp *Eco*RV-*Eco*RI fragment of Ag63 in *Sma*I-*Eco*RI cleaved pGEX-3X (63), a 1010-bp *Eco*RI fragment of Ag361 in *Eco*RI cleaved pSj10 (361), a 763-bp *Eco*RI fragment of Ag16 in *Eco*RI-cleaved pSj10 (16) or a 1317-bp *Eco*RI fragment of RESA in *Eco*RI-cut pGEX-3X (R). The position and sizes (kDa) of M_r markers (M) are indicated.

McNeilage and X. Li, personal communications), a fragment of the α-chain of the murine T-cell receptor (G. Morahan, personal communication), and murine interleukin 4 and GMCSF (unpublished data). Sizes of fusion proteins range from 32 to 84 kDa.

In a minority of cases, purification has been unsuccessful and these failures are all attributable to insolubility of the fusion protein. Insolubility is a frequent characteristic of foreign proteins expressed in *E. coli* (Marston, 1986) and in this context it is surprising that the majority of GST fusion proteins are wholly or partly soluble. Little is known about the factors responsible for insolubility (Marston, 1986) but in several instances insolubility of GST fusion proteins is associated with the presence of strongly hydrophobic regions and elimination of these regions greatly increases stability and/or solubility (our unpublished data; C. Langford, in preparation; G. Peterson, personal communication). Other insoluble fusion proteins either contain many charged residues or are larger than 100 kDa. Insoluble fusion proteins can nevertheless be purified by affinity chromatography if they are solubilised in 1% Triton X-100, 1% Tween 20, 10 mM dithiothreitol or 0.03% SDS, since these conditions do not disrupt binding to glutathione-agarose (our unpublished data). Purification of other insoluble fusion proteins must be by conventional methods (Marston, 1986) unless the polypeptide can be expressed in several fragments that each form a soluble fusion protein. Insolubility has sometimes been associated with increased protein stability in *E. coli* (Cheng et al., 1981), but not in other cases (Schoemaker et al., 1985). In general both insoluble and soluble GST fusion proteins are stable and where direct comparison is possible, the stability of a polypeptide expressed as a soluble GST fusion is similar to that expressed as an insoluble β-galactosidase fusion (unpublished data).

Despite the amino acid sequence identity of 42% between Sj26 and the rat GST Yb_1 isozyme, no immunological cross-reactivity has been observed between Sj26 and rabbit GST isozymes (Smith et al., 1986) and good antibody responses have been generated against the foreign polypeptide portion of fusions in immunised mice, rabbits and sheep (unpublished data). Although experience is limited, responses appear to be as good as or better than those to equivalent β-galactosidase fusions, perhaps reflecting the smaller size of the GST carrier (26 kDa compared with 116 kDa). Responses to Sj26 vary in different mouse strains (Davern et al., 1987) and similar variation is observed in the response to polypeptides expressed as fusions with GST (our unpublished data).

(c) Protease cleavage of purified fusion proteins

The utility of the pGEX vectors for the production of foreign polypeptides in *E. coli* would be increased

if, following purification, the GST carrier could be removed from fusion proteins by cleavage with site-specific proteases. This approach has been successful for some fusion proteins containing the recognition site of factor X_a (Nagai and Thorgersen, 1984) or collagenase (Germino and Bastia, 1984), but has sometimes been ineffective (Allen et al., 1985) perhaps due to the insolubility of fusion proteins or the presence of denaturing reagents.

Oligodeoxynucleotides encoding the cleavage recognition site of thrombin (Chang, 1985) or factor X_a (Nagai and Thorgersen, 1984) were introduced immediately 5′ to the multiple cloning site of pGEX-1 generating the plasmids pGEX-2T and pGEX-3X respectively (Fig. 1b). Insertion of a 580-bp *Rsa*I-*Eco*RI fragment of the Ag63 cDNA (Bianco et al., 1986) between the *Sma*I and *Eco*RI sites of pGEX-2T resulted in the expression of a 43-kDa fusion protein that could be purified on glutathione-agarose (Fig. 4A). Incubation of this protein with thrombin led to the production of two fragments, one the GST carrier, and the other a 22.5-kDa portion of Ag63 (Fig. 4A). Efficient cleavage occurred within 30 min and at an enzyme-to-substrate ratio of 1:500. A small proportion of fusion protein was resistant to cleavage even after incubation for 2 h with a ten-fold higher concentration of enzyme. Similarly, expression of a 555-bp *Eco*RV-*Eco*RI fragment of Ag63 using the pGEX-3X vector resulted in the synthesis of a 43-kDa fusion protein that was cleaved into two fragments by factor X_a (Fig. 4B). Cleavage with factor X_a was slower and less efficient than with thrombin, possibly due to inefficient activation of factor X. Three other pGEX-2 fusions and one additional pGEX-3X fusion have been tested for cleavage by the appropriate protease and in each case efficient cleavage was observed (our unpublished data) suggesting that purified GST fusion proteins are good substrates for site-specific proteolysis, possibly because they are soluble under non-denaturing conditions.

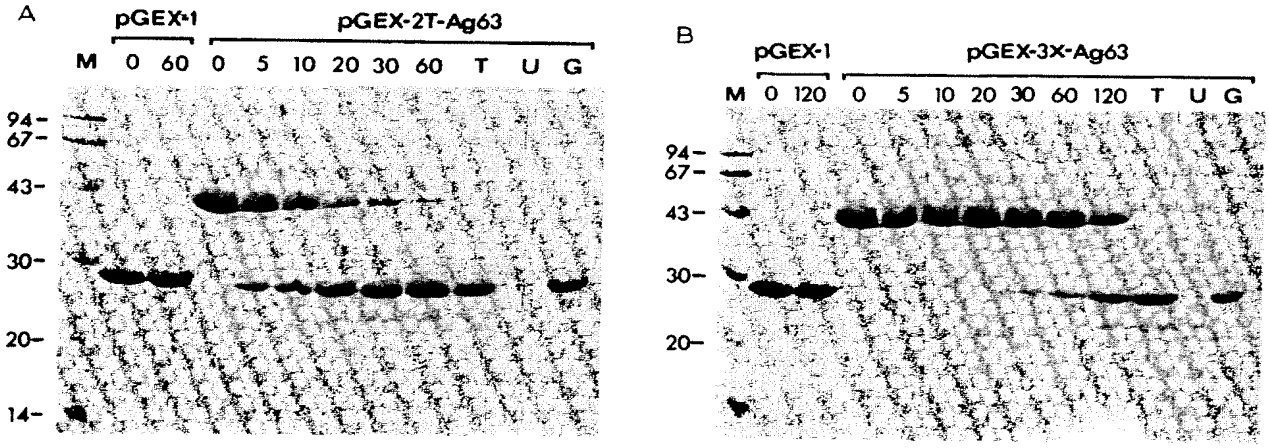

Fig. 4. Protease cleavage of purified fusion proteins. (Panel A) Thrombin cleavage. Purified fusion protein from cells transformed with pGEX-1 or with pGEX-2T containing a 580-bp *Rsa*I-*Eco*RI fragment of Ag63 was incubated with protease at 25°C in elution buffer containing 150 mM NaCl, 2.5 mM $CaCl_2$ and 100 ng human thrombin (Sigma) (Eaton et al., 1986) and approx. 50 μg fusion protein for the number of min (0–60 min) indicated. Lanes T, U and G: cleavage reactions after removal of glutathione by dilution with 40 vols. of MTPBS followed by concentration using a Centricon-10 concentrator (Amicon Corp.); T, total reaction after concentration; U, reaction after incubation with glutathione-agarose beads; G, material bound to glutathione-agarose beads. Samples were analysed by electrophoresis though a 0.1% SDS–13% polyacrylamide gel followed by staining with Coomassie blue. The size (kDa) and position of M_r markers (M) are indicated. (Panel B) Factor X_a cleavage. Purified fusion protein from cells transformed with pGEX-1 or with pGEX-3X containing a 555-bp *Eco*RV-*Eco*RI fragment of Ag63 was incubated for different periods with factor X_a or absorbed with glutathione-agarose after cleavage and analysed as described in panel A. Cleavage with factor X_a was at 25°C in elution buffer containing 100 mM NaCl, 1 mM $CaCl_2$, 500 ng human factor X_a and approx. 50 μg purified fusion protein (Nagai and Thogersen, 1984). Activation of factor X (Sigma) was at 37°C for 5 min in a reaction containing 7 μg factor X, 75 ng activating enzyme, 8 mM Tris · HCl (pH 8.0), 70 mM NaCl and 8 mM $CaCl_2$ (Fujikawa et al., 1972). The apparent excess of released GST carrier following cleavage compared with the amount of the Ag63 polypeptide fragment may reflect differences in the extent of staining of these polypeptides with Coomassie blue.

Neither thrombin nor factor X_a cleave the GST carrier and so after proteolysis both the carrier and any uncleaved fusion protein can be removed from the cleavage reaction by absorption on glutathione-agarose leaving only the purified polypeptide product (Fig. 4). Furthermore, if site-specific proteases can be produced as GST fusion proteins that retain proteolytic activity, it is possible that the protease could also be removed by absorption. This possibility is encouraged by the findings that many fusion proteins are soluble and bind to glutathione-agarose, and that a purified mouse GMCSF-GST fusion protein has biological activity (D. Metcalf, personal communication).

(d) Conclusions

(1) We have constructed a series of pGEX that simplify the purification of foreign polypeptides produced in *E. coli*. Polypeptides are expressed as C-terminal fusions with Sj26 and can be purified under non-denaturing conditions by affinity chromatography on immobilised glutathione. Using batch washing procedures, several fusion proteins can be purified in parallel in less than 2 h with yields of between 1.6 and 15 μg/ml of culture. Almost 50 different polypeptides have been tested in the pGEX system, the majority of which (36/47) are expressed as GST fusion proteins that are soluble and can be purified by glutathione-affinity chromatography.

(2) Protease cleavage sites for thrombin and factor X_a have been introduced at the C terminus of Sj26 so that the GST carrier can be removed from fusion proteins after purification. Six different GST fusion proteins have been tested and shown to be susceptible to specific and efficient cleavage when incubated with the appropriate protease. Further purification of the polypeptide product can be achieved by absorption on immobilised glutathione to remove the GST carrier and any uncleaved fusion protein.

(3) The pGEX vectors contain the over-expressed *lacI*^{q} allele of the *lac* repressor and so expression of GST fusion proteins from the strong *tac* promoter is efficiently repressed until induction with IPTG, regardless of the *lacI* status of the *E. coli* host. This feature, together with the provision of frame-shifted cloning sites, and the high level of expression, ease of purification and efficient site-specific cleavage of GST fusion proteins makes the pGEX vectors convenient for the construction and analysis of cDNA expression libraries, and may also provide an inexpensive alternative to the chemical synthesis of peptides (C. Langford, in preparation).

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